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Characterization and in vitro evaluation of spherulites as sequestering vesicles with potential application in drug detoxification

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Abstract

The aim of the present investigation was to prepare and characterize lecithin spherulites as parenteral drug sequestering agents with potential application in the treatment of drug overdose and chemical poisoning. The spherulites (~200 nm) obtained by controlled hydration and shearing of lipid–alcohol mixtures, revealed unexpected differences in the physical properties of the bilayer when compared to liposomes. Differential scanning calorimetry, 31-phosphorus nuclear magnetic resonance, and pH-sensitive pyranine steady-state fluorescence studies indicated that although spherulites retained the typical bilayer conformation, the arrangement of the phospholipid molecules was perturbed relative to native liposome bilayer. The loosened packing of the phospholipids in bilayers was strongly supported by the relative ease with which spherulites lost the established pH-gradient. This permeability problem was overcome via incorporation of cholesterol in the bilayer. Subsequently, albumin/buffer components were encapsulated in these spherulites and the drug sequestration potential for detoxification application was examined. Citrate pH-gradient spherulites accumulated 75% of external haloperidol while those loaded with ~20% (w/w) albumin were able to take up 45% of haloperidol and 91–95% of taxanes (docetaxel and paclitaxel). In cytotoxicity studies, the competitive internalization of docetaxel by albumin-loaded spherulites resulted in an increase of the IC_{50} value for the free drug. Thus, the spherulite technology could be a versatile approach for actively sequestering toxins in the blood and for reducing the adverse effects by altering the pharmacokinetics and biodistribution of overdosed drugs.

Spherulite; Detoxification; Uptake; Haloperidol; Taxanes

1. Introduction

In the recent years, toxic exposure to drugs has become a serious concern in the western world. For example, deaths resulting from prescribed-drug poisoning are currently recognized as a growing problem in the United States [1,2]. In Canada, approximately 8% of hospitalizations were documented for detoxification treatment following substance abuse [3]. While there are several widely-used and non-specific remedies for such emergencies, such as the administration of activated charcoal, cathartics, diuretics and emetics, none of them can satisfactorily counter the systemic effects of toxins in a rapid manner. In contrast to the non-specific methods of detoxification, specific antidotes act directly by counteracting the chemical toxins and their adverse effects in the body. However, the use of an antidote approach is invariably based on the prior availability of information on the precise nature of the toxin and its mechanism of action. Hence, in view of these limitations there is clearly a need for a versatile and fast-acting approach for reducing the blood and tissue toxin levels of the intoxicated patient. This report describes the application of colloidal particulates in the non-specific detoxification of overdosed drugs.

The idea consists in administering long-circulating particulate colloids shortly after intoxication, which would reduce the bioavailable drug concentration in the body by acting as a sink for toxins. Since it is only the free form of drug that is responsible for toxicity, captured molecules would be unable to exert the toxic effects. Colloids with long-circulating properties can maximize the sequestration and shift the equilibrium towards the central compartment in the body to reduce the tissue levels of toxin (Fig. 1). The application of colloidal

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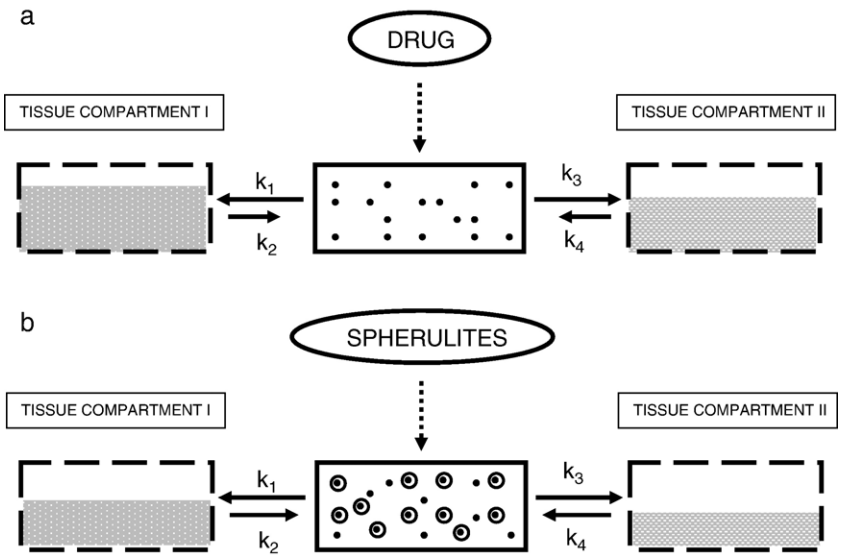

Fig. 1. Pictorial representation of events associated with the chemical intoxication in overdose-cases and the proposed mechanism of action for spherulites. The shaded areas represent the level of drug tissue burden and toxicity following exposure. After exposure, the toxic substance circulating in the blood is free to diffuse into the tissue compartments (a). Administration of spherulites with sequestering potential reduces the free levels of drug in the blood, and consequently, causes the release of tissue-associated substance and its redistribution, thereby alleviating its toxicity (b). Key: ● free toxin; ◉ spherulite-sequestered toxin.

systems for detoxification purposes has already set its momentum with the use of liposomes containing encapsulated chelating agents or enzymes. In the simplest form, liposomes containing chelating agents have been employed to complex metal ions like cadnium [4] or plutonium [5,6] On the other hand, pH-gradients have been used for the selective accumulation of ionizable molecules in lipid vesicles [7]. Other applications involved microencapsulated urease to lower the systemic urea concentration [8]. Furthermore, the initial attempt to antagonize the toxic effects of paraoxan pesticide [9], based on the use of erythrocytes containing the recombinant enzyme phosphotriesterase was subsequently refined by Petrkovics et al. [10], by replacing the erythrocytes with sterically stabilized liposomes. Other recently published investigations for detoxification purposes comprised the use of oligochitosan derivatives bearing electron deficient aromatic rings in the complexation of amitriptyline via π–π interactions [11], as well as nanocapsules with a hexadecane core and a cross-linked polysiloxane/silicate shell for bupivacaine sequestration [12].

In this work, spherulites, a particular type of multilamellar phospholipid vesicles were selected as drug sequestering colloids. These multilamellar spherulites differ from liposomes in that they possess rather more uniformly and closely spaced concentric bilayers of amphiphiles alternating with layers of aqueous medium. Unlike liposomes, spherulites are generally obtained through controlled hydration of sheared lipid/surfactant lamellar phases [13,14]. The unique preparation process imparts them with superior entrapment efficiencies compared to conventional unilamellar and multilamellar liposomes [15,16]. Owing to their onion-structure and constant interlamellar distances, such vesicles were anticipated to perform at least as well as liposomes in terms of the release and protection of encapsulated substances. Unfortunately, the fact that their stability was limited by the undesired permeability of surfactant bilayers recently came to light [14,17]. To circumvent this problem, more complex systems have been designed, such as the dispersion of spherulites in an oil to provide an additional barrier and reduce the diffusion of molecules [18]. However, these multiple w/o/w emulsions also suffered from stability drawbacks due to the expulsion of spherulites from the oil phase. In the present investigation, an attempt was made to prepare surfactant-free biocompatible and biodegradable spherulites, and their sequestration capabilities were fortified either by providing a pH-gradient or by encapsulating albumin in the presence of cholesterol (CHOL). Albumin, a major blood protein, is known to bind several classes of drugs due to a variety of physical interactions. Its affinity generally increases with the hydrophobicity of the molecule. This protein could be exploited in entrapped form to maximize the spherulites sequestration capabilities without causing significant alteration in the blood osmotic pressure following intravenous injection. Since pharmaceutical preparations containing albumin (Abraxane™, Buminate™) are currently marketed for parenteral administration, a safe clinical application of these spherulites for non-specific detoxification purposes could be speculated.

The purpose of this work was to evaluate the potential of using spherulites as drug detoxicants. Sequestration was studied in vitro for three drugs, namely haloperidol, docetaxel, and paclitaxel. The mechanical and permeability properties of the spherulites were modulated by varying the bilayer CHOL concentration. Albumin-loaded and pH-gradient spherulites were prepared in order to maximize the drug uptake into the vesicular aqueous compartments. To understand the bilayer organization and permeability, the spherulites were analyzed by differential scanning calorimetry (DSC), spectrofluorimetry, and phosphorus nuclear magnetic resonance (^{31}P-NMR) spectroscopy. Finally, the drug-sequestration potential of the spherulites was demonstrated by cytotoxicity assay for docetaxel.

2. Materials and methods

2.1. Materials

Phospholipon 90 G (98% soya phosphatidylcholine, SPC) was a gift from Rhône-Poulenc Rörer (Köln, Germany). Distearoyl phosphatidyl ethanolamine polyethylene glycol 2000 (DSPE-PEG_{2000}) and CHOL were purchased from Northern Lipids Inc. (Vancouver, BC, Canada). ^{3}H-labelled haloperidol (12.8 Ci/mmol) was from Perkin Elmer (Woodbridge, ON, Canada). ^{14}C-labelled docetaxel (60 mCi/mmol) and ^{3}H-labelled paclitaxel (60 Ci/mmol) were obtained from American Radiolabeled Chemicals Inc. (St. Louis, MO). Paclitaxel was purchased from Bioxel Pharma Inc. (Sainte-Foy, QC, Canada). Docetaxel was provided by Shanghai Fudan Taxusal New Technology Co. (Shanghai, China). Haloperidol, bovine serum albumin (BSA), (±)-α-tocopherol, pyranine, ferric nitrate, deuterium oxide, ammonium thiocyanate, o-phthaldialdehyde, praseodymium nitrate and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) were procured from Sigma-Aldrich (Oakville, ON, Canada). BCA® reagent and 30% solution of *N*,*N*′bis-methylene acrylamide were purchased from Pierce (Rockford, IL) and BioRad (Hercules, CA), respectively. Dulbecco's modified Eagle's medium, foetal bovine serum (FBS) and antibiotic were from Invitrogen Corp. (Grand Island, NY). Franz diffusion cells and membrane filters were purchased from Cole Parmer (Vernon Hills, IL) and Avestin (Ottawa, ON, Canada), respectively. All other chemicals and solvents were of reagent grade. Water was deionized with a MilliQ purification system (Millipore, Bedford, MA) before use.

2.2. Preparation of vesicles

2.2.1. Preparation of spherulites

Spherulites were prepared by the shearing of the lamellar phase [17,19], using an in-house fabricated apparatus which was comprised of a stainless steel cup (shear cell) and a shear head. A shear-gap of 1 mm was maintained between the cup and rotor head. The cup was mounted into a module with water jackets to maintain the desired temperature. Typically, SPC (200 mg) and CHOL (10–50 mg) were weighed into the shear cell, and 30% (w/w) ethanol containing 10% (w/w) glycerol was added. Then, the rotor head was placed in the cup and rotated at 150 rpm to dissolve the lipids in ethanol at 45 °C. For the incorporation of tocopherol or PEG-DSPE, the excipient was included along with the SPC/CHOL mixture before hydration. The lipid phase was slowly hydrated by the addition of 50 mM phosphate buffered saline (PBS) at pH 7.4 (adjusted to 308 mOsm/L with NaCl) containing or not BSA (50% w/v), or 100 mM citrate/75 mM phosphate buffer pH 3.0 (adjusted to 308 mOsm/L with NaCl) under shear. The formation of lyotropic lamellar phase with birefringent properties was confirmed by polarized light optical microscopy (Axioskop 40, Carl Zeiss Canada, Kirkland, QC, Canada). For this, a small quantity of the sheared sample was placed on a glass slide, covered with a cover-slip and observed through crossed polarizers at a magnification of 100×. Digital images of the samples were acquired using MicroPublisher 3.3RTV camera and processed with Image ProPlus™ (version 5.0) software (Q-Imaging Inc., Burnaby, BC, Canada). To obtain spherulites, the lamellar phase was gradually diluted with the buffer under shear, which was finally passed through a 0.45-μm membrane filter to remove any lipid particles. Free albumin was removed by dialyzing the formulation against 0.5 L of PBS buffer for 7 days (dialyzing buffer changed every 24 h) at 4 °C with stirring, using 100-nm pore size polycarbonate membranes (Millipore). The phospholipid, CHOL and albumin content were assayed by colorimetry using the ferric-ammonium thiocyanate [20], phthaldialdehyde [21] and BCA™ protein assay reagents, respectively.

2.2.2. Preparation of liposomes

Liposomes were prepared by the film hydration method [22]. Typically, SPC along with other components were dissolved in a mixture of chloroform:methanol (2:1 *v/v*) and the lipid film obtained by rotary-evaporation was subjected to an additional period of vacuum for 12 h. Subsequently, the film was hydrated with the required buffer to obtain the multilamellar liposomes of the desired lipid concentration. To prepare unilamellar liposomes, the multilamellar vesicles were extruded through polycarbonate 0.2-μm membrane filter (LiposoFast extruder, Avestin, Ottawa, ON, Canada) at room temperature.

2.3. Size measurements

The mean diameter and size distribution were determined by dynamic light scattering in back scattering mode with a Nano-ZS Zetasizer (Malvern Instrument Ltd., Malvern, Worcestershire, UK). CONTIN program was used to extract the size distributions from the auto-correlation functions.

2.4. Gel electrophoresis

Chemical stability of the encapsulated albumin was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Molecular weight markers, albumin standards and spherulite–protein samples were reduced prior to loading on 7.5% resolving PAGE by the standard method [23]. The separated bands were stained with Coomassie blue, scanned under trans-illuminating white light using MultImage™ plate reader, and analyzed by ChemImager™ 5500 software (Alpha Innotech Corp., San Leandro, CA).

2.5. DSC studies

Thermal scans were acquired for the liposomes and spherulites suspended in 0.9% (w/v) NaCl to reveal any differences in the bilayer organization. Calorimetric measurements were carried out using DSC 2910 calorimeter (TA Instruments Inc. New Castle, DE), calibrated with indium. Samples placed in the aluminum pans were sealed with a lid having pin holes. They were cooled at 5 °C/min to −50 °C, held isothermally for 30 min and heated at 2 °C/min to 80 °C under a flow of helium. All data were analyzed using TA Universal Analysis software version 2.5 H.

2.6. ^{31}P-NMR analysis

Extruded liposomes and spherulites of similar sizes (180–210 nm) were prepared in 0.9% NaCl as described in section 2.2 to obtain a final lipid concentration of 50 mg/mL. ^{31}P-NMR spectra were recorded on a Bruker Avance 300 spectrometer (Bruker, Billerica, MA) operating at 121.45 MHz in a 5-mm broad band probe with proton decoupling at 25 °C, as previously reported [24]. Each sample was suspended in 10% D_2O and 5000–5200 transients were accumulated with a pulse length of 14 μs and 1 s relaxation delay. The free induction decays were processed using an exponential line broadening of 100 Hz prior to Fourier transformation. To obtain information on the distribution of phospholipid molecules, measurements were also performed with 7.5 mM praseodymium nitrate ($PrNO_3$) as shift reagent.

2.7. pH-gradient stability studies

For the measurement of relative stability of trans-membrane pH-gradients of both liposomes and spherulites, fluorescent properties of entrapped pyranine were monitored. Extruded liposomes and spherulites containing pyranine were prepared by the methods described in Section 2.2 using a citrate hydration buffer (10 mM, pH 3.0, adjusted to 308 mOsm/L with NaCl) containing 5 mM pyranine. To remove the free dye from the extravesicular bulk phase as well as generate a pH-gradient, the preparations were dialyzed against 50 mM phosphate buffer (pH 8.0, adjusted to 308 mOsm/L with NaCl). Dialysis was performed at 4 °C (100-nm pore size membrane) with the dialyzing medium replaced every 12 h for 4 days. Prior to fluorescence measurements, lipid suspensions were diluted with pH 8 PBS to a final lipid concentration of 0.5 mg/mL. Excitation scans were obtained on an AMINCO Bowman™ Spectrofluorimeter (Thermo Spectronic Instruments, Rochester, NY) at an emission wavelength of 520 nm with slit width of 2 nm. All formulations were stored at 4 °C and measurements were performed at 5 °C to minimize the flux across membrane.

2.8. In vitro drug uptake studies

The uptake of haloperidol, docetaxel or paclitaxel into spherulite/liposome formulations was determined by following the time course of drug transfer from the donor to the acceptor compartment using jacketed Franz diffusion cells. During the uptake studies, the temperature was maintained at 37±0.2 °C using a circulating water bath and cells were continuously stirred at 600 rpm. The

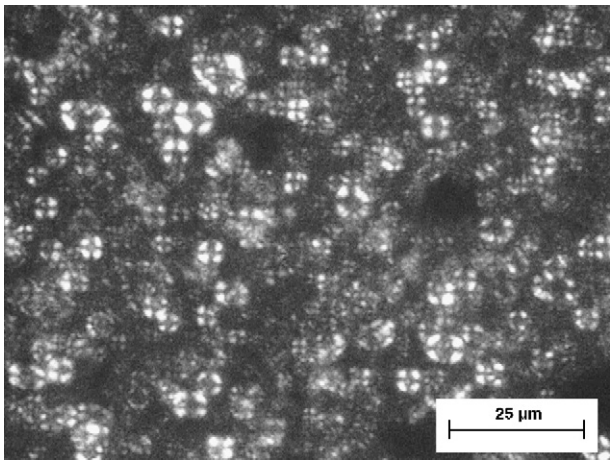

Fig. 2. Polarized light optical microscopy of spherulites (magnification 100×) prepared in the presence of ethanol showing a large number of Maltese crosses in the sheared sample. The characteristic birefringent texture was observed for all spherulite formulation.

concentration of drug was 40 ng/mL and the solutions were spiked with 0.04 μCi/mL $^{3}H/^{14}C$-labelled drug. Prior to the set-up of the diffusion cells, both the membrane and O-rings were incubated in drug-containing PBS (pH 7.4). Then, the required quantity of formulation (500 μL, pH 7.4) was introduced into the lower compartment through the side port. The donor (sampling) compartment was physically separated from the acceptor compartment containing formulation by a 50-nm pore diameter membrane. At the zero time point, the volumes in the sampling and acceptor compartments were adjusted to 0.8 and 4.8 mL, respectively, to ensure the same drug concentration in both compartments. At pre-determined time intervals, 20-μL aliquots were withdrawn from the sampling compartment, mixed with 5 mL of UltimaGold™ and analyzed by radioactivity counting. The rate of disappearance of drug from the sampling compartment was plotted as a function of time and correlated to the drug uptake.

2.9. Cell culture experiments

Cytotoxicity studies were performed on B16F10 cancer cells to assess the uptake of docetaxel into albumin–spherulites by the MTT proliferation assay [25]. B16F10 melanoma cell-line was obtained from the American Type Culture collection (ATCC Rockville, MA) and grown in the Dulbecco's modified Eagle's medium supplemented with 10% FBS and 1% (w/v) penicillin–streptomycin in a humidified atmosphere at 37 °C with 5% carbon dioxide in air. After the cells reached 85–90% confluence, they were trypsinized and seeded in 96-well plates at a density of 1000 cells/well. They were allowed to adhere for 24 h, and the medium in wells was replaced with 100 μL of fresh medium containing increasing concentrations of docetaxel (0.1–100 nM). An equal quantity of either saline or spherulites suspended in saline was added, and the plates were incubated at 37 °C for 15 h. After treatment, the contents of each well were removed by aspiration. 100 μL of fresh medium and was added and the cells were allowed to grow for 48 h. At the end of this period, MTT reagent (10 μL of 5 mg/mL) was added; the formazan produced was solubilized by the addition of 100 μL of 15% (w/v) SDS in 0.01 N HCl and the absorbance was measured at 470 nm with a Safire™ plate reader (Tecan Austria GmbH, Salzburg, Austria).

3. Results

3.1. Preparation of spherulites

In this report, spherulites were prepared from phospholipids dissolved in ethanol containing 10% (w/w) glycerol in the absence of any surfactant. This finding should be beneficial from the formulation point-of-view since many surfactants approved for parenteral use are not completely free from side-effects. Thus, the absence of surfactants would allow the administration of large quantities of phospholipid spherulites in the clinical setting while dealing with heavily intoxicated subjects. Shearing of SPC-ethanol lamellar phase under controlled hydration at 45 °C yielded spherulites with an average diameter of 200 nm (polydispersity index <0.25). When examined by polarizing microscopy, the concentrated phase of spherulites contained a polarization cross with the arms parallel to the plane of polarization, indicating that the molecules were arranged in layers (Fig. 2).

Spherulite formulations loaded with BSA (a surrogate for human albumin) were prepared to maximize the sequestration properties of the vesicles. For the spherulites having a lipid membrane composition of SPC/CHOL ratio 200/10, 200/30 and 200/50 (w/w), the BSA loadings reached 1.7, 13.1 and 19.6% (w/w), respectively. In addition to the membrane composition, other factors that were studied included, shearing temperature and pH. Increasing the shearing temperature from 45 to 50 °C as well as reducing the pH from 7.4 to 4.7 (iso-electric point of albumin) did not improve the entrapment of BSA (data not shown). SDS-PAGE stability studies showed that the entrapped albumin was protected from degradation in all formulations and remained chemically intact after storage at 4 °C for at least 15 days (Fig. 3).

In the past, investigations were focused on the preparation of spherulites either from surfactant or phospholipids + surfactant bilayers and the published literature showed no report where spherulites were obtained from 100% lecithin. Due to these differences, it was pertinent to understand the molecular organization of the system. Lipid molecules could, in fact, organize into novel arrangements which would have a direct impact on their performance. Hence, the membrane properties

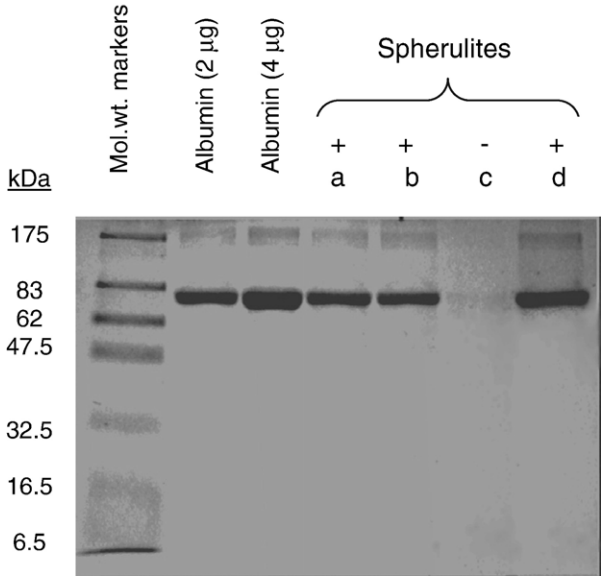

Fig. 3. SDS-PAGE data of spherulites with (+) and without (−) entrapped BSA. Key: Spherulites without CHOL (a), with CHOL (b), and with CHOL and 5 mol % DSPE-PEG (c, d).

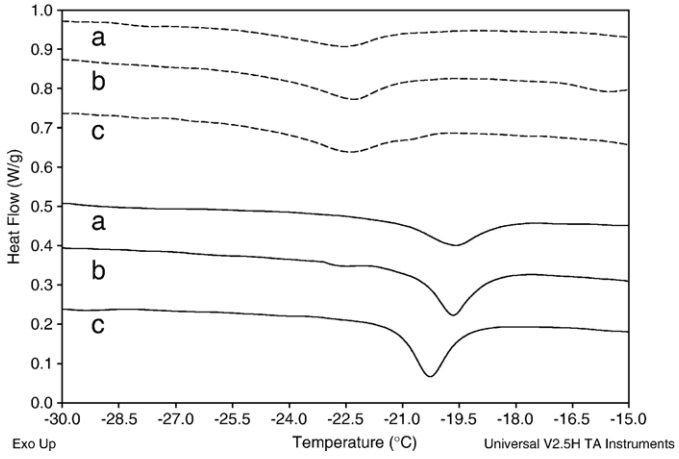

Fig. 4. DSC traces of liposomes (solid lines) and spherulites (broken lines) during the heating phase of a cool–heat cycle. The endotherms were recorded at a heating rate of 2 °C/min. All formulations were suspended in saline and were in the size range of 180–210 nm. Key: (a) SPC/CHOL=200/50, (b) 200/30, (c) 200/0 (w/w).

of the spherulites were examined by DSC, NMR spectroscopy and pyranine-fluorescence studies.

3.2. DSC studies

DSC is an extensively investigated, sensitive, and non-perturbing tool used to study the thermotropic phase transitions of lipid membranes. Lipid bilayer vesicles display a number of phase transitions of which, the gel-to-fluid transition, characterized by a disordering of the acyl chains, is especially diagnostic of membrane organization. This transition is generally observed for the unsaturated phospholipids below the freezing point of water (Fig. 4). Upon heating, the control extruded liposome formulations exhibited the main phase transition (T_m) at −20.3, −19.7 and −19.6 °C for formulations with SPC/CHOL ratios of 200/0, 200/30 and 200/50, respectively. On the other hand, for the same sequence of lipid ratios, spherulites displayed lower T_m at −22.4, −22.3 and −22.6 °C, respectively. Liposomal bilayers exhibited a relatively sharp and symmetric endothermic peak while that of spherulites was asymmetric and broad. The peak widths at half-height were in the range of 1 to 1.5 and 2.3 to 2.8 °C for liposomes and spherulites, respectively. As shown in Fig. 4, the variation of heat flow associated with the gel-to-liquid crystalline transition decreased progressively in intensity with higher CHOL content. Thus, CHOL seemed to be inserted in the spherulite bilayers while exerting an effect similar to that in the liposomes. Furthermore, DSC studies revealed that $\Delta T_{1/2}$ and T_m were essentially the same for BSA encapsulated spherulites as that for albumin-free spherulites (data not shown).

3.3. ^{31}P-NMR analysis

^{31}P-NMR spectroscopy provides valuable information on the organization of lipid bilayer vesicles due to the sensitivity of the conformation and dynamics of the phosphate nucleus with respect to the surrounding environment. All formulations showed a spectrum shape intermediate to an isotropic and powder pattern, typical of phospholipid vesicles with a hydrodynamic radius around 200 nm (Fig. 5) [26–28]. The low intensity isotropic signals (0 ppm) superimposed on the regular powder patterns could be attributed to the presence of tiny, rapidly tumbling vesicles or could be associated with other

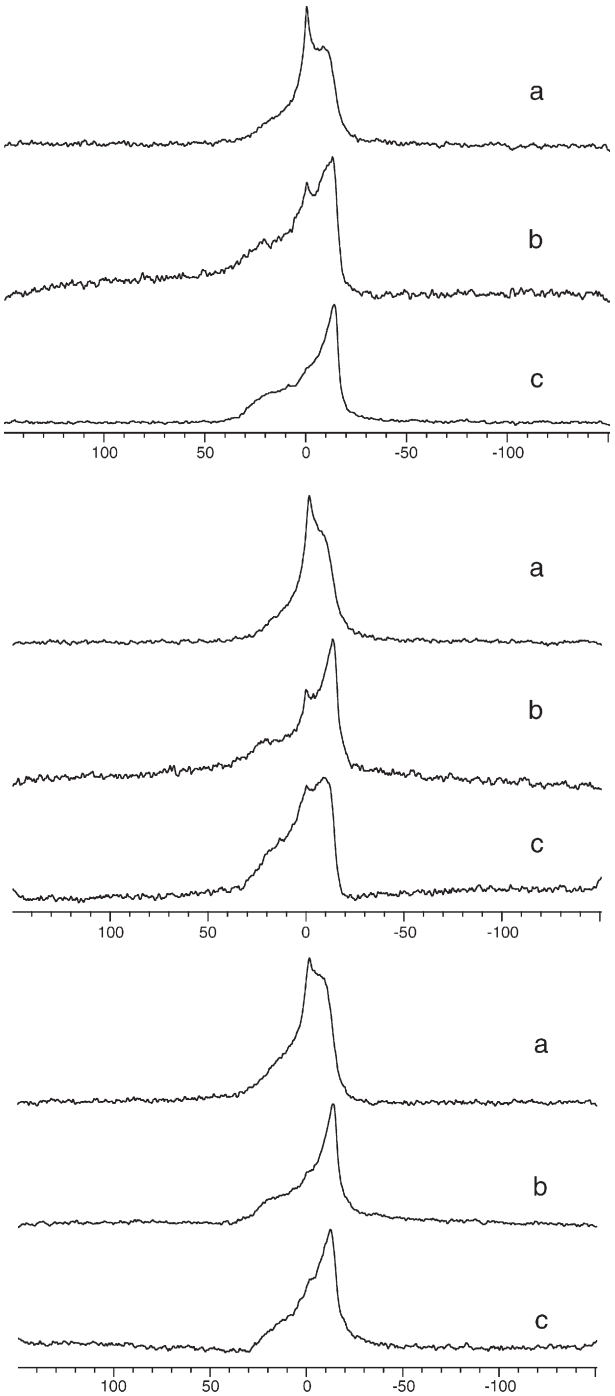

Fig. 5. ^{31}P-NMR spectra for liposomes (a), spherulites (b) and BSA-loaded spherulites (c). All formulations were suspended in saline and were in the size range of 180–210 nm. Scale in ppm. Key: Top: SPC/CHOL 200/0, middle: 200/30, bottom: 200/50 (w/w).

phases (such as cubic phase) with isotropic motions on the NMR time scale. Compared to liposomes, the ^{31}P-NMR spectra of spherulites were systematically closer to powder patterns. This trend was observed for all formulations investigated, independent of CHOL content. CHOL had little effect on the spectra of liquid-crystalline bilayers, as previously observed [29]. To investigate the possibility that albumin could interfere with spherulite formation, ^{31}P-NMR analysis was also performed on BSA-loaded spherulites at various lipid to CHOL ratios (Fig. 5c). The resulting NMR spectra were similar to those obtained without BSA. This indicates that the spherulites could encapsulate albumin both in the presence and absence of CHOL, without any significant perturbation of the bilayer structure.

The ratio of phospholipid molecules on the outside to that on the inside of vesicles (i.e. lamellarity), was determined by relative peak area after shifting the exterior phosphate resonances downfield (~30 ppm) by the addition of 7.5 mM Pr^{3+}(Table 1). For liposomes, the area ratio between the signal of the outer to inner (O/I) monolayers ranged between 0.46 and 0.51. Spherulites exhibited a lower O/I ratio, ranging from 0.35 to 0.37 in the absence of BSA, suggesting an increased lamellarity. From Table 1, it is also observable that the O/I ratio decreases following the entrapment of albumin in CHOL-containing vesicles.

3.4. pH-gradient stability studies

A number previously published reports have demonstrated the uptake of basic drugs into liposomes bearing an acidic intravesicular pH environment [30–32]. With time, the established pH-gradients can gradually dissipate due to the exchange of protons and counter-ions across the bilayer. This leads to a loss in the efficiency of internalization of those drugs whose transfer is pH-gradient driven. Thus, monitoring the pH-gradients across bilayers provides an indication of the performance of the formulation and an assessment of its stability. Pyranine is a pH-sensitive fluorescent acidic probe (pK_a ~7.22) which has been explored for steady state measurements when trapped within the aqueous interiors of vesicular systems [33]. In the excitation spectra, λ_{max} at 405 and 450 nm are assigned to the unionized and ionized forms of pyranine, respectively [34].

Table 1
^{31}P-NMR O/I ratios for SPC liposomes and spherulites in the presence of 7.5 mM $Pr(NO)_3$ shift reagent

Formulation	Ratio[a] (O/I)
SPC:CHOL (w/w)	
Liposomes	
200:0	0.49
200:30	0.46
200:50	0.51
Spherulites	
200:0	0.35
200:0 (BSA)	0.40
200:30	0.37
200:30 (BSA)	0.32
200:50	0.37
200:50 (BSA)	0.22

Mean vesicle diameter and polydispersity index were 200 nm and 0.25, respectively.
[a] Ratio of external to internal phospholipid molecules.

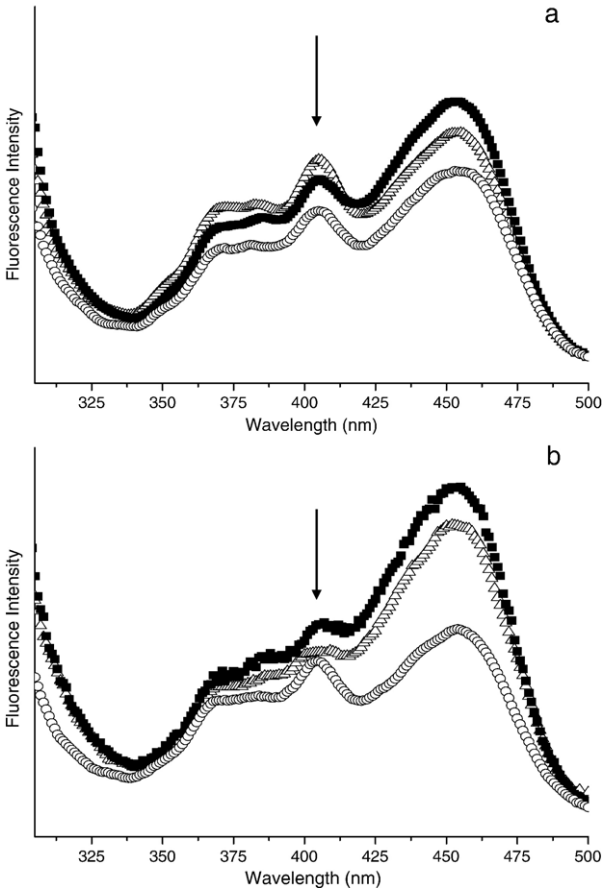

Fig. 6. Fluorescence excitation spectra of encapsulated pyranine in liposomes (a) and spherulites (b). Pyranine was encapsulated in formulations at internal pH 3.0 citrate buffer and dialyzed against pH 8 buffer for 4 days at 4 °C prior to the acquisition of the spectra. Note the differences in the intensity of the excitation peak at 405 nm (indicated by arrows). See text for detailed explanation. Key: (△) SPC; (♦) SPC/Tocopherol: 200/22; (○) SPC/CHOL: 200/50 (w/w).

Pyranine was entrapped in both liposomes and spherulites of varying membrane compositions at an internal pH of 3. The vesicles were challenged against an external pH of 8 for 4 days, after which the fluorescence spectra were monitored. Figs. 6a and b show the excitation spectra of entrapped pyranine for liposomes and spherulites, of which the membrane constitution was either SPC alone, SPC/tocopherol (200/22) or SPC/CHOL (200/50). Since peroxidized bilayers are known to be highly leaky, tocopherol was also incorporated in the bilayers to find out if lipid peroxidation was a major factor in promoting the permeability of spherulites prepared at 45 °C [33]. All liposomal formulations, irrespective of their composition, exhibited a distinct peak at 405 nm in the excitation spectra which corresponds to the unionized pyranine species. This peak was of lower intensity in the SPC and SPC/tocopherol spherulites, indicating the low unionized/ionized ratios for entrapped

Table 2
Emission ratios [a] (E_m450/405) of entrapped pyranine in pH-gradient [b] liposome and spherulite formulations after a 4-day dialysis period

Formulation	E_m450/405 ratio		
	SPC	SPC/tocopherol	SPC/CHOL
Liposomes	1.13	1.41	1.25
Spherulites	1.76	1.76	1.20

[a] The ratios were obtained from excitation spectra. A high E_m450/405 ratio indicates a high ionized/unionized ratio for pyranine.
[b] 100 mM citrate buffer pH 3.0.

pyranine. However, the spherulites retained this peak when CHOL was incorporated into the membrane. The ratios of emission intensity at 450/405 (E_m450/405) is an indicator of the relative acidic to alkaline shift in pH within the interior of vesicles following the challenge period [34]. These ratios were 1.13, 1.41, 1.25 and 1.76, 1.76, 1.20 for SPC, SPC/tocopherol, SPC/CHOL liposomes and spherulites, respectively (Table 2). The noticeably high E_m450/405 values for the spherulites bearing SPC and SPC/tocopherol compositions indicate a shift in pH accompanied by neutralization of the acidic compartment. These results suggest that even at considerably high levels, tocopherol was not effective in diminishing the permeability and rules out lipid peroxidation as a major factor for the high bilayer permeability in spherulites. Further, the E_m450/405 ratio of spherulites was comparable to that of liposomes when CHOL was incorporated within the bilayer indicating reduced permeability of the bilayer to the flux of ions [32,33].

Table 3
In vitro uptake of haloperidol, docetaxel and paclitaxel by SPC/CHOL spherulites [a] in PBS buffer at pH 7.4 and 37 °C

CHOL (mg)	PEG-DSPE (mol%)	Internal pH	Entrapped BSA [b]	Uptake [c] (%)
Haloperidol				
10	0	7.4	−	31.0 (1.5)
10	0	7.4	+	31.0 (1.1)
30	0	7.4	−	21.0 (1.6)
30	0	7.4	+	38.6 (3.1)
50	0	7.4	−	18.9 (1.7)
50	0	7.4	+	47.0 (1.6)
50	5	7.4	+	45.4 (0.3)
50	0	3.0 (75 mM phosphate)	−	52.5 (1.9)
50	0	3.0 (100 mM citrate)	−	75.2 (1.3)
Docetaxel				
30	0	7.4	−	77.0 (1.4)
30	0	7.4	+	94.4 (2.3)
Paclitaxel				
30	0	7.4	−	75.9 (1.4)
30	0	7.4	+	91.5 (1.6)

All data are mean, $n=4$ (SD).
[a] SPC content was set to 200 mg in all formulations. Mean vesicle diameter and polydispersity index were 200 nm and 0.25, respectively.
[b] Albumin encapsulation was 1.7, 13.1 and 19.6% (w/w) for SPC/CHOL: 200/10, 200/30 and 200/50, respectively.
[c] Uptake studies were conducted at drug and lipid concentrations of 40 ng/mL and 3 mg/mL, respectively.

Table 4
In vitro uptake of haloperidol by SPC/CHOL spherulites [a] in albumin-containing PBS buffer at pH 7.4 and 37 °C

CHOL (mg)	PEG-DSPE (mol%)	Internal pH	Entrapped BSA	External albumin (%)	Uptake [b] (%)
30	0	7.4	−	1	13.2 (2.1)
30	0	7.4	+	1	25.4 (2.8)
50	0	7.4	−	1	18.1 (2.2)
50	0	7.4	+	1	24.7 (2.0)
50	5	7.4	+	1	28.6 (0.8)
50	0	3.0 [c]	−	1	68.1 (1.7)
50	0	3.0 [c]	−	3	53.5 (1.5)

All data are mean, $n=4$ (SD).
[a] SPC content was set at 200 mg in all formulations. Mean vesicle diameter and polydispersity index were 200 nm and 0.25, respectively.
[b] The uptake studies were conducted at drug and lipid concentrations of 40 ng/mL and 3 mg/mL, respectively.
[c] Citrate buffer 100 mM.

3.5. Uptake studies

The relative sequestration capabilities of various formulations were evaluated through uptake studies using the Franz diffusion cells. Uptake of the weak base haloperidol into spherulites was monitored during a period of 4 h in the absence (Table 3) and presence of external albumin (Table 4) or FBS (Table 5). All formulations exhibited a first-order uptake profile suggesting that the kinetic process was concentration-gradient driven. Moreover, these profiles showed no apparent lag time and a time-of-50% uptake in the range of 20–30 min, suggesting a rapid drug uptake (data not shown). In the absence of entrapped albumin, spherulites showed the highest uptake of haloperidol (31%) when the SPC/CHOL ratio was 200/10 (Table 3). It decreased to 21% and 18.9% at SPC/CHOL ratios of 200/30 and 200/50, respectively. It is also interesting to note that, at SPC/CHOL ratio of 200/10, the incorporation of BSA made no significant contribution to the uptake ($p<0.05$, unpaired t-test). This situation was reversed upon entrapment of BSA at higher CHOL levels (SPC/CHOL 200/30 and 200/50). A consistent feature of albumin–spherulites was an enhanced capacity to take up haloperidol as the CHOL content was increased. (Table 3). It was found that the incorporation of 5 mol% DSPE-PEG_{2000} (a lipid used to prolong the circulation times of lipid vesicles) into SPC/CHOL 200/50 spherulites, had no impact on the uptake of haloperidol (Table 3). Subsequent attempts with pH-gradient SPC/CHOL 200/50 spherulites

Table 5
In vitro uptake of haloperidol into SPC/CHOL (200/50) pH-gradient [a] liposomes and spherulites in the absence and presence of FBS at 37 °C

Formulation	Uptake [b] (%)	
	No FBS	50% FBS
Multilamellar liposomes	69.5 (1.1)	27.5 (0.8)
Extruded liposomes	70.9 (0.7)	33.4 (0.9)
Spherulites	75.2 (1.3)	38.3 (1.2)

All data are mean, $n=4$ (SD).
[a] 100 mM citrate buffer pH 3.0.
[b] The uptake studies were conducted at drug and lipid concentrations of 40 ng/mL and 3 mg/mL, respectively.

(internal pH of 3, 75 mM phosphate or 100 mM citrate) showed much higher uptake values. They ranged from 52.5 to 75.2% (Table 3), and were significantly higher than for the albumin-encapsulated systems ($p<0.05$, unpaired t-test). Owing to its basic nature, haloperidol can freely cross the membrane in the non-ionized state and accumulate in the acidic compartment of the pH-gradient vesicles.

In order to mimic physiological conditions, the sequestration of haloperidol into spherulites was evaluated in the presence of albumin in the external medium (Table 4). For BSA-spherulites, at 1% external albumin, the uptake was reduced from 38.6 to 25.4%, and from 47.0 to 24.7% at SPC/CHOL ratios of 200/30 and 200/50, respectively. Similar decreases were observed for BSA-free spherulites. Haloperidol uptake into PEGylated spherulites also reduced substantially and was comparable to that of the uncoated formulations. For citrate pH-gradient spherulites, the competitive effect of external albumin had less impact on the uptake (Table 4). Additionally, when the stability of spherulites was assessed, the vesicle size remained unchanged for at least 2 months after preparation. The uptake of haloperidol was then re-examined for the pH-gradient spherulites stored under refrigerated conditions. After a period of 45 days, it decreased from 52.5% to 42.4% for phosphate pH-gradient spherulites while it remained unchanged for citrate pH-gradient spherulites ($p>0.05$, unpaired t-test). These differences in stability (phosphate vs. citrate) of vesicles might be due to the different permeability values for phosphate and citrate ions across the bilayers.

The capture of haloperidol by spherulites (pH 3, citrate gradient) was then compared to multilamellar (780 nm) and extruded (180 nm) liposomes of identical membrane composition (SPC/CHOL 200/50) (Table 5). These pH-gradient liposomal formulations showed uptake values of 69.5 and 70.9%, respectively, in PBS. When 50% FBS was added to the medium, the uptake values were lowered by 60, 53, and 49%, for multilamellar liposomes, extruded liposomes, and spherulites, respectively. The higher uptake by spherulites ($p<0.05$, ANOVA), both in the presence and absence of FBS may be attributed to a higher encapsulation of citrate buffer thereby contributing to the higher net sequestration of haloperidol.

Finally, the uptake of the non-ionizable drugs, docetaxel and paclitaxel was examined into BSA-spherulites. pH-gradient spherulites were not employed in this case because of the neutral character of these drugs. Due to the highly hydrophobic nature and affinity for bilayers, the sequestration of taxanes was higher than that of haloperidol for a given composition of spherulites. Drug uptake at a SPC/CHOL ratio of 200/30 was 77.0% for docetaxel and 75.9% for paclitaxel. Upon entrapment of albumin, these values increased to 93.7 and 91.5%, respectively ($p<0.05$, unpaired t-test, Table 3). Clearly, the incremental uptakes could be attributed to the presence of albumin and its binding property which was not altered during formulation.

3.6. Cytotoxicity studies

To complement the in vitro uptake data, cell viability studies were conducted on docetaxel-sensitive melanoma cells either by exposing the cells to the free drug alone or the free drug in the presence of spherulites. PEGylated BSA-spherulites were chosen in view of the slightly higher uptake observed for haloperidol in an albumin environment, as well to minimize the interaction between the vesicles and the cells. A significant decrease in the cytotoxicity of docetaxel was noted when the cells were exposed to the drug in the presence of albumin-loaded PEGylated spherulites made of SPC/CHOL 200/50 (Fig. 7). The calculated IC_{50} value for docetaxel alone was 5.2 (±1.0) nM, while it increased to 8.0 (±1.1) and 14.0 (±2.5) nM in the presence of 0.75 and 1 mg/mL spherulites, respectively ($p<0.01$, ANOVA). The higher viability of cells at higher spherulite concentrations clearly implies a fall in the levels of free drug in the culture medium due to the partitioning of docetaxel into the spherulites.

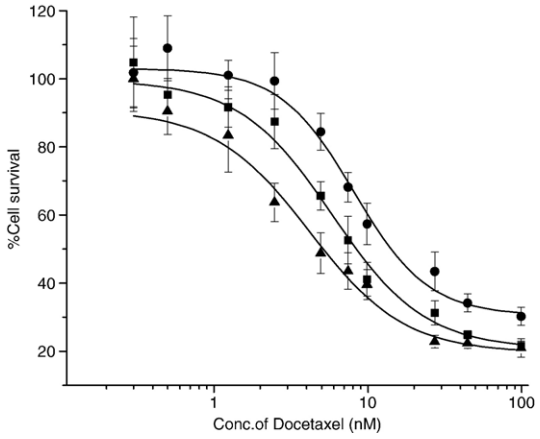

Fig. 7. Survival of B16-F10 cells as a function of docetaxel concentration in the absence (▲) and presence of 0.75 (■) and 1 mg/mL (●) BSA-loaded spherulites (SPC/CHOL: 200/50 coated with 5 mol% DSPE-PEG).

4. Discussion

An interesting aspect associated with the shearing of lyotropic lamellar phase comprise phospholipid and surfactant mixtures is the formation of well organized lamellar structures called spherulites [14,15]. Spherulites have been proposed as an alternative to liposomes for achieving higher entrapment of water-soluble molecules [13–15,17]. During pre-formulation investigations, we discovered that bilayers containing surfactants were highly leaky. In an attempt to produce surfactant-free spherulites, ethanol was found to be a viable option. Lipids dissolved in ethanol formed spherulites upon controlled hydration and shear. Ethanol was subsequently eliminated from the formulation by dialysis. These surfactant-free spherulites, which are expected to possess a toxicological profile similar to phospholipid liposomes, were further explored for application to detoxification purposes.

The spherulites were examined by DSC and compared to extruded liposomes of similar size. The observed differences in thermal transition events, i.e. a shift in T_m and a broadening of the transition profile, could be ascribed to a loss of cooperativity [35,36]. During the phase transition, the melting process is a

cooperative event due to the close packing of lipid chains and thus gives rise to a sharp peak in the DSC thermogram. However, when the cooperativity is disturbed due to perturbation in the packing of the bilayer, the lipid acyl chains undergo a more gradual disordering, which leads to the broadening of the phase transition. Steady-state fluorescence measurements with entrapped pyranine also revealed that under similar conditions, spherulites devoid of CHOL failed to maintain an established pH-gradient, while liposomes were successful. Hence, the increased permeability of the bilayer confirmed the perturbations in the packing of the spherulite membrane. In addition to the changes in T_m, the subtle differences in ^{31}P-resonance peak profiles between spherulite bilayers and liposomes could be attributed to decreased vesicle tumbling rates. These were likely caused by the larger lipid mass contained in spherulites. The proposed loosened packing of lipid molecules in spherulites may be related to (i) an osmotic stress imposed on the bilayers by the non-uniform distribution of entrapped solutes during lipid organization and hydration, and/or (ii) the presence of packing defects in the bilayer structure related to the fabrication of spherulites from sheared lamellar phases [13,33,36,37]. Further studies are needed to verify these hypotheses.

In this work, the application of spherulites for a generalized and non-specific detoxification approach was verified in vitro by incorporating albumin or acidic buffers, and by examining the accumulation of non-ionizable (taxanes) and ionizable (haloperidol) molecules. As described above, DSC and fluorescence data together revealed that lipid acyl chains are loosely packed and weaker interactive forces prevail in spherulites. Hence, the rigidizing properties of CHOL on these bilayers were exploited, and CHOL amounts were systematically varied to improve the spherulites sequestration capabilities. The condensing effect of CHOL on the liquid-crystalline phospholipid bilayers results in tighter molecular packing thereby decreasing the permeability to hydrophilic molecules and the penetrability of water into the bilayers [29,38,39]. Since the total quantity of drug sequestered is a summation of the amount solubilized in the bilayers and the internal aqueous phase, it is not surprising that the uptake of haloperidol decreased with increasing CHOL amounts in the lipid bilayer. The decreased affinity of lipid membranes for haloperidol with increasing CHOL concentration was previously studied by Sarmento et al. [40]. They reported a 7-fold decrease in the apparent partition coefficient for a 1.5-fold increase in CHOL bilayer content. In contrast, a noticeable feature of the albumin-spherulites was the higher internalization of haloperidol with increasing CHOL/SPC ratio. This can be explained by the physical changes in the bilayer introduced by the steroid during membrane organization (see NMR data), consequently increasing the loading of albumin and subsequent drug sequestration. This hypothesis is supported by the ^{31}P-NMR experimentation which points out to the increased lamellarity of albumin-spherulites, and consequently the entrapment of albumin, as an important effect of CHOL on these bilayers (Table 1).

A noticeable feature of pH-gradient spherulites is their ability to take up higher amounts of haloperidol compared to albumin-spherulites, especially in the medium mimicking the physiological environment. An indirect evidence in favor of the pH-gradient spherulite approach can be drawn from a study conducted with egg phosphatidylcholine/CHOL liposomes exhibiting a trans-membrane pH-gradient (300 mM citrate, pH 4) [7]. The ability of these liposomes to alter the pharmacokinetics of doxorubicin and increase the maximum tolerated dose in mice correlated well with the uptake under in vitro conditions. Accordingly, it could be stated that pH-gradient driven potential is superior to that of the albumin encapsulated system for the uptake of basic drugs. On the other hand, the formulations based on the protein encapsulation approach are potentially useful sequesters for membrane-permeable, non-ionizable toxins which do not respond to pH such as taxanes. However, it needs to be mentioned that under in vivo conditions, the detoxifying properties of albumin-spherulites could be reduced by the competitive binding of the toxin to the blood components. Thus, in order for this approach to be successful in the clinic and for achieving a rapid detoxification action, the albumin-spherulites would need to be dosed by intravenous infusion at levels that are required to cause displacement of already bound drugs in favor of spherulites. In this regard, the countered inhibitory action of the free drug on cell proliferation by PEGylated albumin-spherulites, as well as the spherulite dose-dependent increase in IC_{50} value of docetaxel in the cytotoxicity studies, is a fine illustration of their detoxification potential. Hence, it is anticipated that the specific properties imparted to the vesicles by PEG and CHOL, namely stealthiness and membrane rigidity, would collectively reduce their elimination by the mononuclear phagocyte system in vivo, thereby allowing the spherulites to circulate long enough to scavenge and sequester toxins or overdosed drugs.

5. Conclusion

To our knowledge, this is the first demonstration of application of surfactant-free spherulites as a novel tool in the armamentarium for poisoning treatment. The sequestration capabilities of spherulites have been augmented either by the encapsulation of albumin or by the establishment of a trans-membrane pH-gradient. The former approach would be a versatile tool for the internalization of almost any toxin or drug with affinity for albumin, while the latter formulations would rapidly respond to basic molecules. It was possible to substantiate the sequestration capabilities of spherulites by virtue of their ability to reduce the cytotoxic effects of docetaxel on cancer cells. Regardless of the fact that spherulites resemble liposomes in terms of bilayer constitution, dye-entrapment studies revealed that the bilayers of spherulites are apparently more permeable than liposomes. In this regard, CHOL was found to be a suitable membrane component to prevent the leakage of contents across the inherently permeable spherulite bilayers. Since the physical properties of bilayers govern the interaction and movement of molecules across the lipid membrane, as well as influence the in vivo circulation and disposition of vesicles, further studies are warranted to better comprehend these related phenomena and to improve the therapeutic application of these systems.

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